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The objective of this two year project was to identify protein structure domains participating in proton transport and membrane voltage interactions by the plasma membrane H^+-ATPase from Saccharomyces cerevisiae. H^+-ATPase mutants (pma1) were generated by random and site-directed mutagenesis techniques that caused a deploarization of the cellular membrane potential. All pma1 mutant enzymes were active in proton transport, although one mutant, Gly158-->Asp, appeared to be partially uncoupled from ATP hydrolysis. Three loci, one within a putative transmembrane domain (Gly158) and the other two (Ser368, Pro640) within putative membrane/cytoplasmic interface domains, were found to cause the most prominent effect on cellular membrane potential. To better understand how membrane voltage effects the H^+-ATPase, a new procedure was developed to produce large and sustained membrane potentials in reconstituted proteoliposomes. The results of this project will serve as a foundation for probing electrogenic proton transport by the H^+-ATPase with the eventual goal of developing a structural model for ion translocation. Keywords: Cations, Phosphorus Hydrolases, (AW)

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INTRODUCTION

The objective of this project was to define protein structure domains of the plasma membrane H^+-ATPase from yeast that are involved with proton transport and membrane voltage interactions. Our approach has been to isolate mutants of the H^+-ATPase (pma1) that alter electrogenic proton transport by the enzyme and then identify specific amino acid alterations.

The yeast H^+-ATPase is an electrogenic proton pump that plays a vital role in nutrient uptake and intracellular pH regulation, and the gene encoding this enzyme, PMA1, is essential for growth. The cellular importance of the H^+-ATPase requires that viable pma1 mutants can only arise from mutations resulting in partially active or conditionally inactive enzymes. In collaboration with Dr. James E. Haber (Brandeis University), we described a positive selection procedure, based on resistance to the positively-charged antibiotic hygromycin B, for isolating partially defective H^+-ATPases (McCusker, J.E., Perlin, D.S. and Haber, J.E. 1987 Mol. Cell. Biol. 7, 4082-4088). The original screen resulted in the isolation of 75 pma1 mutants and the first year of this project was spent characterizing the biochemical properties of these mutant enzymes. The second year was spent identifying PMA1 genetic defects, as well as characterizing the proton transport properties of mutant enzymes. The mutant H^+-ATPases showed diverse biochemical phenotypes (Km, Vmax, pH optima, inhibitor sensitivity, etc.); yet, despite these differences, all of the mutant enzymes shared the common property of inducing strong depolarizations of cellular membrane potential (Perlin, D.S., Brown, C.L. and Haber, J.E. 1988 J. Biol. Chem. 263, 18118-18122). The pma1 mutants were viewed as important for understanding electrogenic proton transport by the H^+-ATPase.

FINAL REPORT

First Year

1. Properties of pma1 mutants. Most pma1 mutants were unable to tolerate acid loading conditions which included growth at low external pH or growth in the presence of weak acids. The mutants were also very sensitive to NH_4^+ and medium osmotic pressure; these phenotypes were all complemented by plasmid-associated normal PMA1. Intragenic complementation of pma1 mutants suggested that the H^+-ATPase is likely to be a dimeric enzyme.

2. Biochemical properties of pma1 mutants. Expression and assembly of the H^+-ATPase appeared normal in the majority of pma1 mutants since wild type levels of intact enzyme, M_r=100,000, were found. Three types of kinetic defects resulting in a decreased K_m and/or V_{max} were found (Fig. 1); enzymes from two strains, pma1-105 and pma1-141 which were growth inhibited by low pH, showed a precipitous decline in V_{max} below pH 6.5. The H^+-ATPase is strongly inhibited by vanadate and three mutants enzymes, pma1-105, pma1-141 and pma1-147 were found to be vanadate-insensitive. Intragenic second-site supression of these primary mutations led to the isolation of partial revertants with restored vanadate sensitivity. Vanadate-insensitive enzymes formed normal phosphorylated intermediates but appeared to show

differences in steady-state levels of E_1 and E_2 conformational intermediates during catalysis.

3. Whole cell transport behavior of pma1 mutants. It was found that net proton efflux, as measured by whole cell medium acidification in the presence of 25 mM KCl, was nearly identical for wild type and pma1 mutant cells. However, in the absence of added KCl, the initial rate and final extent of net proton efflux for wild type was considerably less than that of the pma1 mutants. Changes in proton leak pathways were not considered likely since passive proton conductance and intracellular buffering capacity were unaltered in the mutants. The cellular membrane potential was identified as an essential factor in regulating proton fluxes and was found from [^{14}C]-tetraphenylphosphonium distribution studies to be strongly depolarized in pma1 mutants (Fig. 2). Depolarization of the membrane potential also helped explain resistance of pma1 mutants to yeast killer toxin. The action of yeast killer toxin has been linked to a hyperpolarized membrane state.

The important finding that hygromycin B-resistant pma1 mutants showed defects in the cellular membrane potential suggested that hygromycin B was an effective selective agent for isolating depolarized cells. Changes in cellular membrane potential were a direct consequence of mutations within PMA1 that altered the H^+-ATPase and one exciting possibility was that pma1 mutant enzymes had altered charge-transfer properties. In the second year of this project, it was important to characterize the genetic defects associate with the various pma1 mutants and examine in more detail the transport properties of mutant enzymes.

Second Year

1. **Genetic defects of pma1 mutants.** The most severely affected mutant alleles displaying membrane potential defects were cloned and sequenced. Single base-pair changes were found in pma1-105, pma1-147, pma1-141 and pma1-114 that resulted in amino acid substitutions of Ser368-->Phe, Pro640-->Leu, Ser368-->Phe and Gly158-->Asp, respectively. According to a recent proposed topographical model for the H^+-ATPase (Serrano, R. 1988 Biochim. Biophys. Acta 947, 1-28), Gly158 is expected to be buried within a transmembrane helical domain, while Ser368 and Pro640 lie within a large catalytic domain. Both residues are predicted to be close to the membrane/cytoplasmic interface. In the course of cloning and sequencing pma1 mutants, six amino acid substitutions, Pro74-->Leu, Val209-->Ile, Lys444-->Met, Ser479-->Phe, Ala480-->Val and Ala836-->Ser were identified in the Y55 wildtype background strain which had no apparent effect on enzyme function.

2. Importance of Ser368 in membrane potential depolarization. A mutation affecting Ser368 was found to cause one of the most severe phenotypes. To further examine the influence of this residue on steady-state membrane potential formation, a detailed revertant and site-directed mutagenesis approach was used to create numerous amino acid substitutions. The results indicated that replacement of Ser368 with Phe, Val or Leu led to a marked depolarization of cellular membrane potential. Interestingly, Phe, Val and Leu substitutions resulted in a range of biochemical properties. The most

prominent effect was seen by their sensitivity to the mechanistic inhibitor vanadate (Fig. 3). The Phe368 mutant is vanadate insesitive, the Val368 mutant enzyme is vanadate sensitive and the Leu368 mutant enzyme is intermediate in sensitivity. Wildtype enzyme, a second vanadate-insensitive mutant allele, Leu640, and a vanadate-sensitive mutant allele. Asp158 are included for comparison.

3. Proton transport by mutant enzymes. Our initial assessment of proton transport by mutant enzymes relied on whole cell measurements of H^+-ATPase-dependent medium acidification. To more precisely define proton transport by the mutant enzymes, we developed a purification and reconstituion procedure that results in recovery of reconstituted enzyme at greater that 85% purity with nearly 100% of its initial actvity. When reconstituted, all mutant enzymes formed ATP-induced pH gradients, as determined by fluorescence quenching of the pH gradient probe acridine orange. Proton transport in K^+-loaded vesicles was found to be optimal in the presence of valinomycin which eliminated any transient membrane potential formation by allowing for compensating charge movement. When mutant enzymes, as illustrated for pma1-105 (Fig. 4A), were allowed to form transient membrane potentials in the absence of valinomycin, there was a pronounced decline in the apparent rate of proton transport relative to wildtype. The addition of valinomycin restored pH gradient formation to its optimal level. These effects are suggestive of an altered voltage sensitivity by the mutant enzyme. It was also observed that proton transport by pma1-114 mutant enzyme was significantly less than that of wildtype or other mutant enzymes with identical ATP turnover rates (Fig. 4B). The possiblity was raised that this represented a partially uncoupled mutant.

4. An in vitro assay for assessing membrane voltage effects. In an effort to analyze the effects of membrane voltage on mutant enzymes more precisely, an in vitro system was developed which allows large and sustained membrane potentials to be generated in liposomes. In this assay system, which was developed at the suggestion of Dr. H. Ti Tien (Michigan State University), electron flow from ascorbate (inside liposomes) to ferricyanide (outside liposomes) is mediated via the electron carrier TCNQ. With this procedure, we are able to generate relatively large and sustained interior positive membrane potentials. Fig.5A illustrates that membrane potential formation was readily followed by the potential-dependent probe Oxonol V. In this example, membrane potential formation was initiated by the addition of ferricyanide to proteoliposomes containing ascorbate, K^+-gluconate and TCNQ. The membrane potential decayed with time and was fully collapsed by the addition of valinomycin. By varying the lipid composition, the decay kinetics could be altered considerably (Fig. 5B). In the presence of 80% E. coli lipids and 20% phosphatidylserine the rate of decay in reconstituted vesicles is sufficiently slow to allow measurements of ATP hydrolysis. Preliminary data indicatedn a 50% decline in ATP hydrolysis during maximum potential formation.

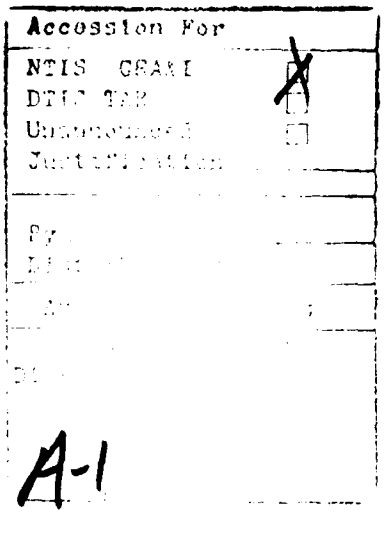
Perspectives and Future goals. The primary goal of this project was to identify protein structure domains of the H^+-ATPase that function in electrogenic proton transport. While it is not yet possible to construct a model for ion translocation and coupling, significant progress has been made in

identifying residues and protein structure domains participating in these reactions. In an effort to probe the proton translocation domain(s) more directly, site-directed and localized random mutagenesis will be used to target residues within the membrane bilayer. By generating numerous mutants that effect only partial catalytic reactions, it should be possible to propose concrete structural models for electrogenic proton translocation. This highly directed approach is currently being pursued in ONR Grant #NOOO14-89-J-1792 entitled: Mechanism for H^+-transport and membrane voltage interactions in a yeast H^+-ATPase.

PUBLICATIONS

Perlin, D.S., Brown, C.L. and Haber, J.E. 1988 Membrane potential defect in hygromycin B-resistant pma1 mutants of Saccharomyces cerevisiae. J. Biol. Chem. 263, 18118-18122

Perlin, D.S., Harris, S.L., Seto-Young, D. and Haber, J.E. 1989 Defective H^+-ATPase of hygromycin B-resistant pma1 mutants from Saccharomyces cerevisiae. J. Biol. Chem., in press.



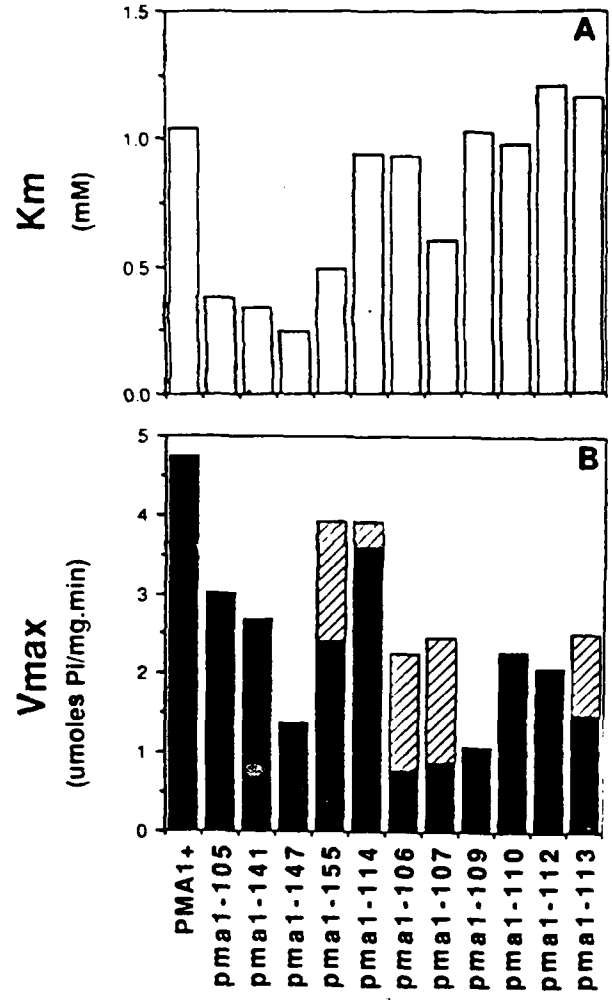


Fig. 1. Kinetic properties of mutant enzymes. Kinetic parameters Km (panel A) and Vmax (panel B) were determined for mutant enzymes at pH 6.5. The cross-hatch area in panel B represents Vmax values normalized to control levels of intact enzyme.

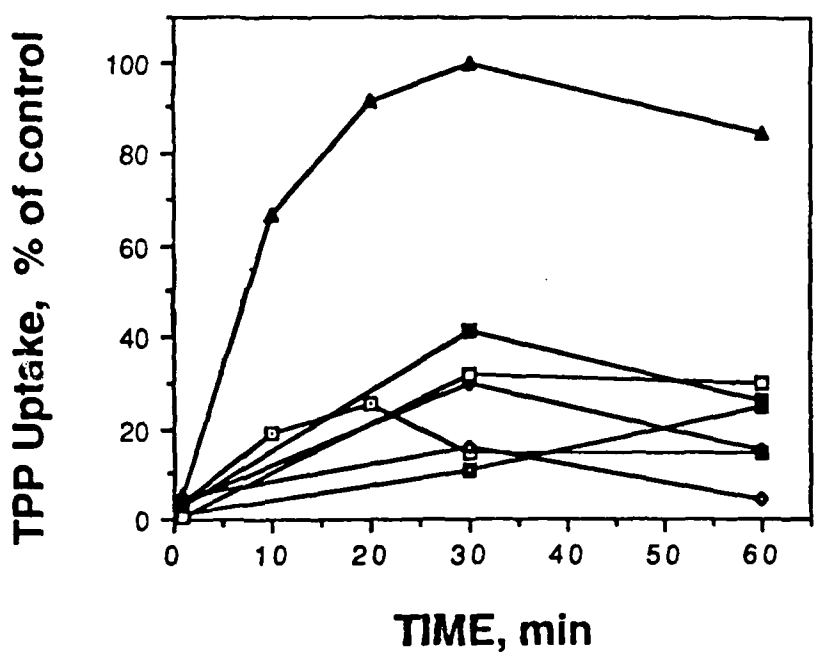


Fig. 2. Uptake of [^{14}C]-TPP by pma1 mutants. Steady-state uptake of [^{14}C]-TPP by wild type (▲) and pma1-101 (◆), pma1-105 (▣), pma1-114 (■), pma1-141 (■), pma1-147 (□) and pma1-155 (◇) mutants in the presence of glucose was determined by a rapid filtration assay. Uptake from de-energized cells was subtracted from these plots.

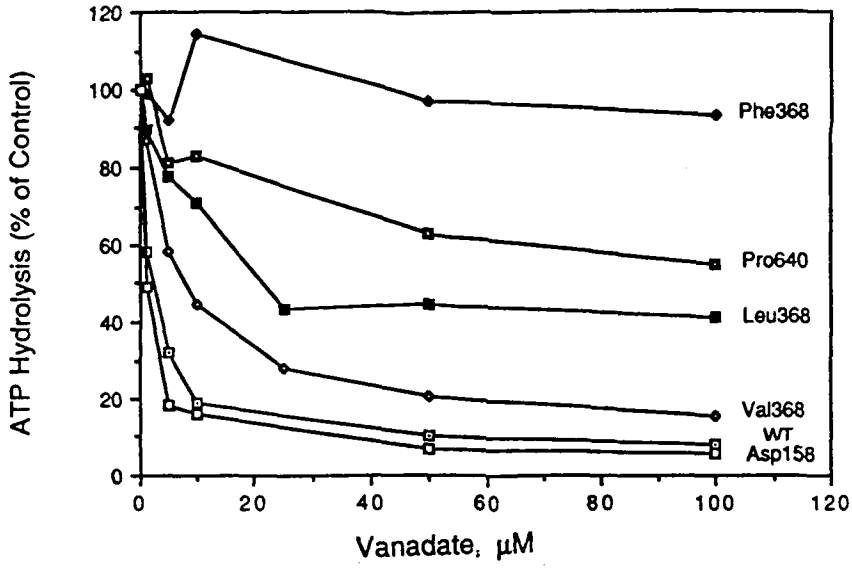


Fig. 3 Effect of vanadate on ATP hydrolysis by mutant enzymes.

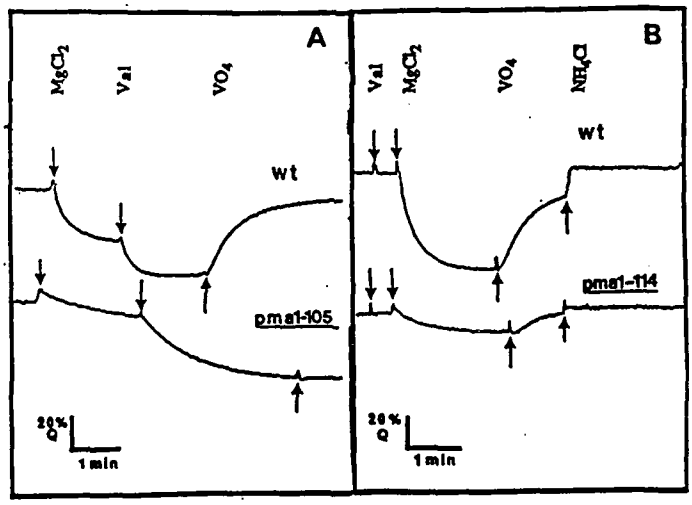


Fig. 4 Proton transport by reconstituted wildtype and pma1 mutant enzymes. The quenching of acridine orange fluorescence was used to assess interior acid pH gradient formation by reconstituted wildtype and mutant enzymes. The reaction medium contained 10mM HEPES-KOH, pH 6.8, 50mM KCl, 5mM ATP and 10μg reconstituted protein. Proteoliposomes were preloaded with 50mM KCl. ATP-linked proton transport was initiated following addition of 5mM $MgCl_2$. All other additions were as indicated (1μM valinomycin; 10μM vanadate; 10mM ammonium chloride).

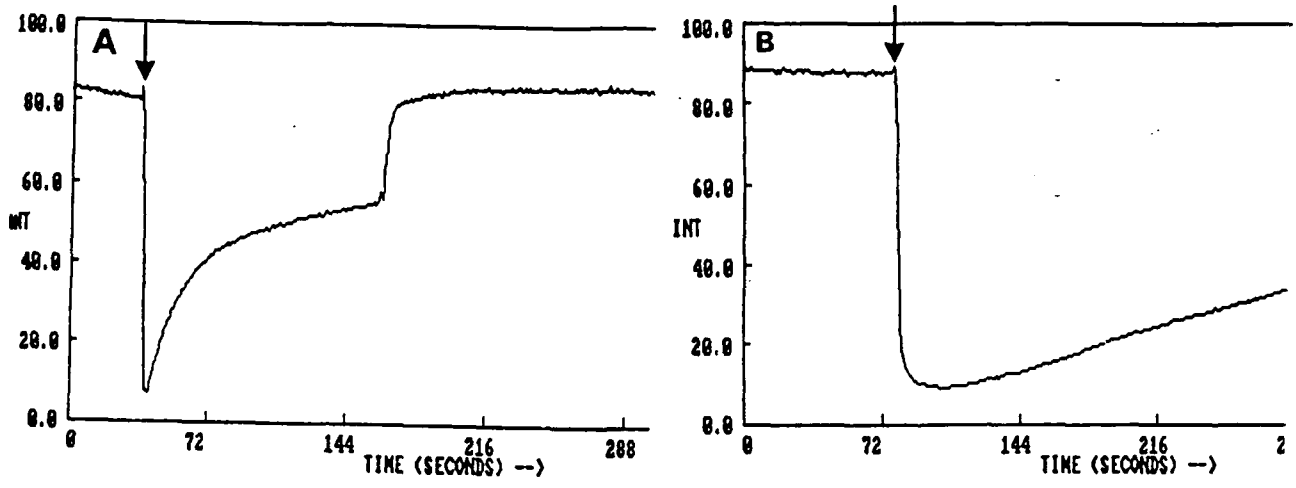


Fig. 5. Induced membrane potential formation in proteoliposomes. The quenching of oxonol V fluorescence was used to assess interior positive membrane potenial formation. Proteoliposomes (10ug) pre-equlibrated with ascorbate, K+ and TCNQ were suspended in medium containing 10mM HEPES-KOH, pH 7.0, 100mM K-gluconate and 1uM oxonol V. Ferricyanide was added to initiated membrane potential formation. Proteoliposomes were prepared with 20% asolectin, 70% E. coli lipids and 10% PS (panel A) or 80% E. coli lipids and 20% PS (panel B).